APPENDIX A

Chapter 3. β-Amyloid as a Target for Alzheimer's Dis as Therapy

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Introduction - One of the cardinal pathological signatures of Alzheimer's disease (AD) is the deposition of the β-amyloid (Aβ) peptide in the cores of senile plaques and in the walls of cerebral blood vessels (1). This 39-42 amino acid peptide is formed from the cleavage of a larger species, the β-amyloid precursor protein (βAPP), and is prone to self-aggregation (2). It is believed that the Aβ peptide plays a key role in the pathogenesis of AD. Some evidence that supports this hypothesis is the following: (a) three familial forms of AD - trisomy 21, βAPP gene mutations, and presenilin gene mutations, all increase the production of Aβ; (b) the most prominent genetic risk factor for AD, which is apolipoprotein Eε4, is associated with increased Aβ deposition; (c) fibrillized Aβ is toxic to neurons in vitro and in vivo; and (d) transgenic mice overexpressing human βAPP develop AD-like cerebral amyloid plaques. One approach for blocking the formation of β-amyloid is to inhibit the polymerization of this peptide. For other amyloidoses, such as AA and AL amyloidosis, it has been shown that blocking or removing the amyloidogenic peptide, can reverse the process. Furthermore, other therapeutic targets, the β- and γ-secretases that cleave βAPP to form Aβ, have not yet been unequivocally identified. Also, Aβ aggregation inhibitors binding to the peptide may block the interactions of Aβ with other molecules involved in AD pathophysiology, including complement proteins, apolipoprotein E, heparin sulfate proteoglycan, RAGE, and scavenger receptors. The inhibition of Aβ aggregation is thus expected to slow or arrest the progress of AD, rather than treating the symptoms of the disease. This chapter will focus on the utilization of biophysical techniques for the elucidation of the actual chemical targets, highlight some of the β-amyloid inhibitors that have been reported over the past few years, and give an update on the progress of the animal models.

BIOPHYSICAL METHODS AND THE SEARCH FOR AMYLOID FIBRILLIZATION INHIBITORS

Rationale - In designing inhibitory molecules to block amyloid fibril formation, it is useful to consider the process as a protein folding problem, in which fibril formation occurs along one or more non-native pathways. The insoluble product accumulates in a relatively stable, and usually biologically disruptive state. Deriving a meaningful structure activity relationship (SAR) requires that the assay targets the rate-limiting intermediate and the compound interacts with the inhibitor in the same way, however the activity is measured. Targeting a kinetically defined, low abundance protein folding intermediate whose presence is rate-limiting, is risky. An inconsistent SAR for a protein folding reaction could be due to a change of the rate-limiting species exposing a different site, or to the existence of multiple binding modes for the inhibitors. Different inhibitors may be identified if the structure of the rate-limiting intermediates vary. Therefore, knowing details of th structures involved in the folding reactions and in th pathological amyloid fibril deposits is important in developing effectiv amyloid fibrillization inhibitors.

Electron Microscopy and X-Ray Diffraction - The regularity of the organization and crossed β-sheet structure of amyloid fibrils are defined histologically by their tinctorial properties with Congo Red birefringence and Thioflavine fluorescent staining. High resolution electron micrographs of fibrils that are either negatively stained with heavy metals or shadowed with gold reveal a common structure for all fibrils, regardless of the primary sequence of the protein or peptide involved (3). Image reconstruction and cryoelectron microscopy reveal further detail in conjunction with X-ray fibril diffraction experiments, and support the existence of twisted helices of β-sheets (4-6). Even with magnetic orientation, amyloid fibrils are not sufficiently ordered to diffract to atomic resolution. X-ray diffraction studies of complexes of Congo Red with pig insulin amyloid fibrils have provided a picture of how this dye could interact with the end product of fibril formation (7-9). Inhibitors that stabilize monomeric or low oligomeric Aβ species may facilitate crystallization of these complexes.

Atomic Force Microscopy (AFM) - Kinetic studies with the Aβ peptide supporting nucleation-dependent fibril growth stimulated a search for the elusive intermediate. Electron microscopy provided some hints of small dimeric and oligomeric species that appeared early in the reaction. The smaller species gives rise to protofibrils which eventually lead to the characteristic twisted, 7 nm diameter filaments (10). Atomic force microscopy, in which a thin carbon nitride tip is dragged or tapped along a surface to reveal the topography, was adapted to biological specimens adsorbed onto the atomically smooth surface of freshly cleaved mica. Images with resolution approaching that of electron microscopy can be obtained with carbon nanotube tips (11). Fixation and staining artifacts are avoided with AFM, and the technique can be accomplished in air or under buffer solution, although only surface adsorbed material is imaged (12). A series of AFM images of Aβ(1-42) and Aβ(1-40) revealed oligomeric and protofibrillar structures that appeared to be precursors to mature amyloid fibrils (13-15). AFM and EM are complementary in characterizing the effects of different fibrillization inhibitors on the structure of reaction intermediates and in distinguishing non-specific clumping from true amyloid fibril formation.

Light Scattering - The scattering of visible light by mature amyloid fibrils (turbidity) frequently registers a lag phase since it only detects relatively large particles (≥400 nm), missing smaller, early intermediates (16). While technically simple and useful for an endpoint determination, multiple intermediate species blocked by inhibitors could remain undetected by turbidity. Inhibitors acting at different points early in the reaction would be indistinguishable. Color absorbance and precipitation of peptide and/or inhibitor further interfere with the interpretation of results.

By contrast, dynamic light scattering (quasi-elastic light scattering, QLS) measurements of scattered light intensity at multiple angles from the incident laser beam is capable of resolving nanometer size macromolecular structures at the expense of sample throughput and a requirement for specialized equipment and data analysis. It gives real-time measurements of scatterers of different sizes and is thus useful for characterizing reaction mechanisms of inhibitors identified by other methods. Small oligomeric species of Aβ have been studied by this technique (17-21). However, precipitation and inhibitor absorbance can interfere with the measurements.

Fluorescence - The extrinsic chromophore, Congo Red, and the fluorophore, Thioflavin T, have been used to monitor amyloid fibril formation for a number of amyloidogenic proteins and peptides (22-24). Some yet unelucidated property of amyloid fibril ß-sheet secondary structure elicits the spectroscopic changes, although not all ß-sheets react, particularly those in nativ proteins (25). Aß does not display a hydrophobic pocket detectable by 1-anilinonaphthalenesulfonic acid (1,8-ANS) binding under physiological conditions, unlike most denatured proteins. Inhibition of Thioflavin

T fluorescence has been used to search for inhibitors of amyloid fibril formation. The major caveats to this approach are compound interference with probe binding and optical artifacts. There is also the potential for false negatives for inhibitors which stabilize small, Thioflavin-positive species. Backup filtration or sedimentation assays for fibril formation can address these issues (26).

Detailed studies of the conformational state of the Aβ peptide have been hampered by the lack of intrinsic, sensitive biophysical probes. The amino acid residue substitution, Y10W, preserves fibril formation as do the substitutions, F4C or G34C, after coupling to synthetic fluorophores. Fluorescence energy transfer experiments between these probes suggest dimerization of the peptide, although unequivocal evidence is lacking (27).

Ultracentrifugation - A powerful approach to characterizing the physical state of intermediates in amyloid fibril formation is by sedimentation velocity measurements during the reaction. Early studies with Aβ provided a tantalizing look at the process, but the specialized nature of the equipment, low sensitivity, and low sample throughput restricts its utility (28). The multiple wavelength, multiple sample Beckman XLA ultracentrifugation system, has made these experiments accessible. Species of all molecular sizes can be followed down to the low micromolar range as long as the species that is being monitored has a relative abundance of 5-10% and the species are stable during the centrifugation run. The formation of amyloid fibrils from transthyretin has been characterized by this approach (29).

Nuclear Magnetic Resonance (NMR) - NMR can be used to determine the solution and solid state conformations of peptides and their state of oligomerization, as well as their association with small molecules. Stringent depolymerization of Aβ(1-40) and Aβ(1-42) preparations yields a metastable monomeric species at physiological pH, low ionic strength, and low temperature, which has been used to study interactions of the monomer with nicotine and melatonin (30-32). Solution phase structure determination depends, in a complex way, upon the exchange rates of inhibitor binding and the rates of structural interconversion of peptide conformations. Solid state NMR has been used to characterize the amyloid fibril structure formed by the Aβ(34-42) fragment (33). Methodological improvements promise to extend the utility of the solid state method to smaller amounts of longer peptides and to the binding of small molecules.

Fourier-Transform Infrared Spectroscopy (FTIR) - FTIR has been used to garner secondary structure information for both soluble and fibrillar Aβ peptides. ^{13}C-Isotope editing of the synthetic C-terminal fragment, Aβ(34-42), localized an abnormal conformational state in the Gly37, Gly38 region (34). Availability of recombinant, selectively isotope-enriched peptides and/or small molecule binders could lead to a resurgence in these types of studies (35). The protein in senile plaques of Alzheimer's Disease brain tissue has been shown by FTIR microscopy to be mostly in an amyloid-like crossed β-sheet conformation (36).
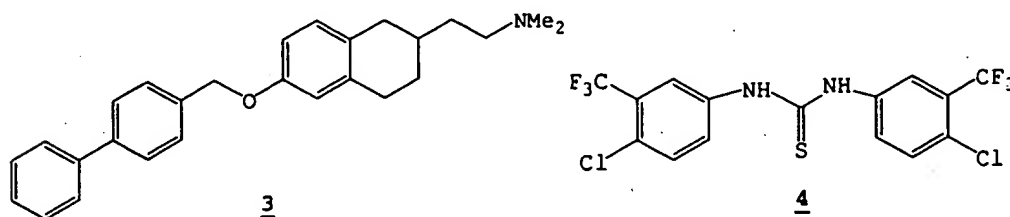
β-AMYLOID INHIBITORS

Secretase Inhibitors - Much of the current research is directed towards the design of inhibitors that block the yet unidentified enzymes, α-, β-, and γ-secretases, that cleave the β-amyloid precursor protein (βAPP) to form Aβ. Inhibition of the β- and γ-secretases should reduce Aβ formation and thus slow the progression of Alzheimer's

Disease. Sequential proteolysis liberates Aβ from βAPP, i.e., β-secretase cleaves at the N-terminus of Aβ, followed by C-terminal γ-secretase hydrolysis (37-39).

Amino acid derivatives, 1 and 2, inhibit the release and/or synthesis of Aβ, and therefore, are claimed as being useful in the prevention and treatment of Alzheimer's (40, 41).
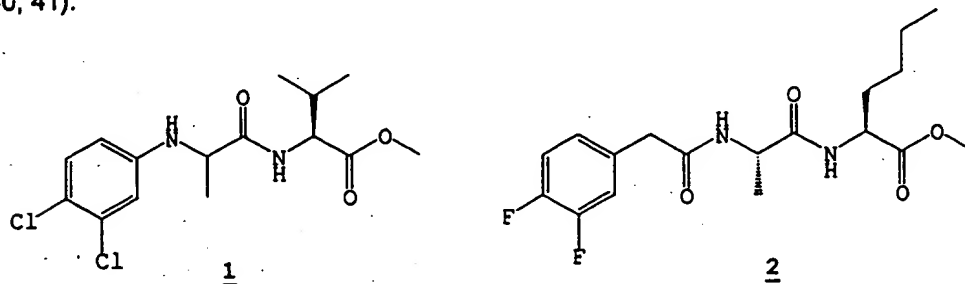

Amine and urea analogs, 3 and 4, have also been claimed in the patent literature as Aβ production inhibitors (42, 43). It was reported that 3 inhibits production and/or secretion of Aβ1-40 and Aβ1-42 by 74% and 75%, respectively, relative to control, whereas 4 inhibits Aβ production activity in cells at 10μg/mL (IC$_{50}$=2.8μM).

Most of the compounds reported are claimed for the inhibition of β-amyloid protein production rather than as secretase inhibitors. Another such series of compounds are hydroxy-hexanamide derivatives, such as 5, which are expected to be effective in treating patients suffering from the cerebral accumulation of β-amyloid protein, such as in Alzheimer's Disease (44).
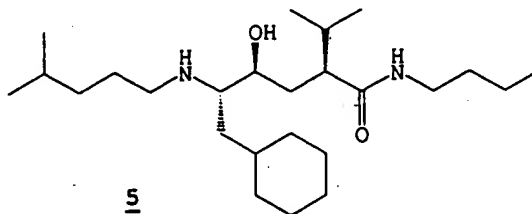

Aggregation Inhibitors – Aβ is postulated to be a key player in the series of events culminating in neurodegeneration and clinical dementia (1). Previously reported antiaggregation inhibitors such as IDOX and Rifampicin are being improved (45). Imino-aza-anthracyclinone derivatives of IDOX, such as 6, have recently been claimed as useful for the prevention of amyloid formation and/or the induction of amyloid degradation in peripheral and CNS amyloidoses (46).
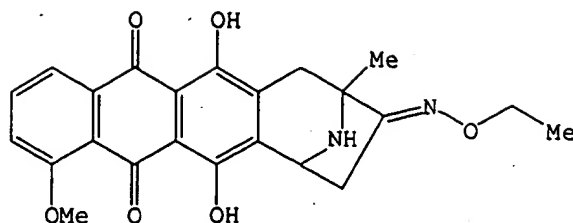
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Other high molecular weight molecules, such as Amphotericin B, porphyrins and phthalocyanines have also been reported to have anti-aggregation effects (47, 48).

Short, modified peptides derived from the Aβ sequence, such as **7**, have been shown in some assays to prevent fibril formation and Aβ toxicity (49).
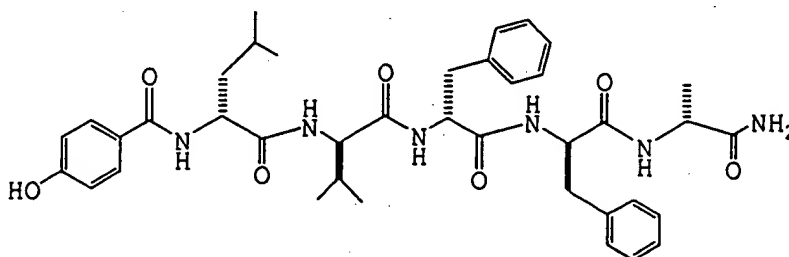

7

Small molecule inhibitors from various chemical classes have recently been reported and reviewed (50, 51). These compounds inhibit or reduce *in vitro* aggregation of Aβ peptides, although the peptide species being inhibited is unknown. Somewhat similar in structure to Congo Red **8**, the amyloid staining agent that inhibits the formation of Aβ(1-40) fibrils, are naphthyl monoazo derivatives, such as **9** (52). These types of molecules are claimed for imaging amyloid and blocking amyloid deposition (53).
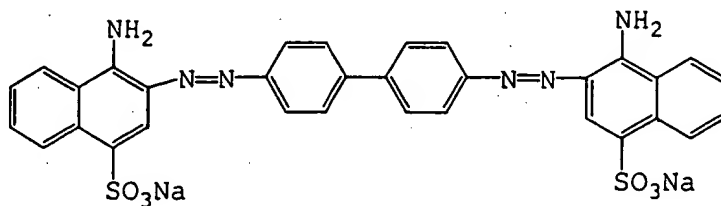
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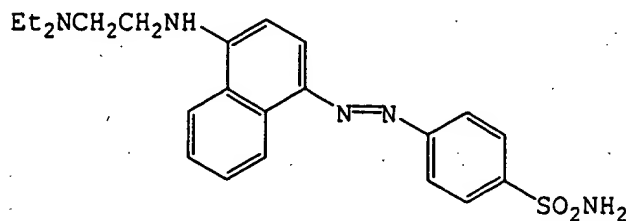
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Other molecules with similar properties towards β-amyloid are acridinones and benzothiazole derivatives, such as **10** and **11** (54, 55).


10   **11**
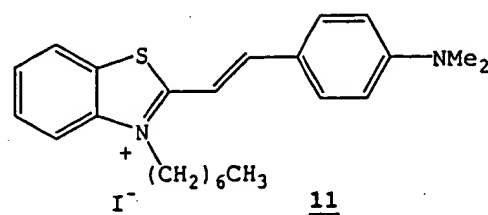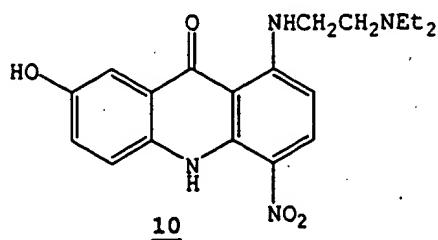
Quaternary N-alkyl ammonium salts, such as hexadecyl-N-methylpiperidinium bromide **12** (HMP), form stoichiometric complexes with the Aβ-peptide well below their critical micellar concentration, suggesting that these types of compounds are not acting solely as detergents (56). Simple disulfonates and disulfates, such as 1,4-butanedisulfonic acid disodium salt **13**, interfere with splenic amyloid deposition in a mouse model of AA amyloidosis (57).

12   **13**
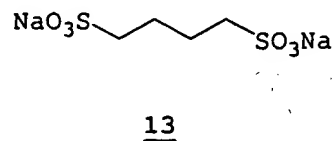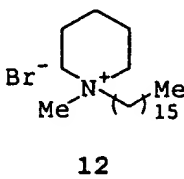
Finally, nicotine **14** and melatonin **15** and some of their derivatives have been reported to be effective at high concentrations in preventing Aβ fibril formation under specified conditions (58, 59). From NMR studies and the apparent stoichiometry, these molecules appear to interact primarily with the monomeric peptide. This is the only instance in which the nature of the peptide that is interacting with an inhibitor is known.

14   **15**
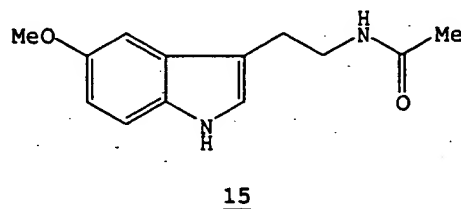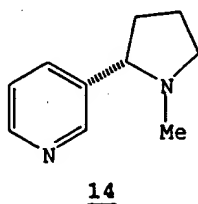
INHIBITION OF Aβ TOXICITY

One of the ephemeral properties of the Aβ peptide is its apparent toxicity in some cellular and animal models (60-62). The irreproducibility among different laboratories and between different lots of various synthetic Aβ peptides, depending on its past history of the peptide is matched only by the different toxicity assays used and the variety of cellular systems affected. A particular conformational state of the peptide seems to be required and a low abundance non-fibril intermediate species may be responsible for the peptide's effects, possibly through a surface receptor(s) (63-67). While toxicity has been a popular target, the physiological response can be

ameliorated by so many things that increase cellular tolerance to insults that the specificity needed to develop an inhibitor SAR becomes difficult to achieve (68). Secretase inhibitors, antioxidants, aggregation inhibitors, and metabolic enhancers could all diminish Aβ toxicity. Aβ-specific central nervous system animal models that demonstrate toxicity are not readily available.

ANIMAL MODELS

Testing inhibitors of β-amyloid production or deposition ultimately requires a suitable animal model. Fortunately, with the advent of transgenic mice bearing various forms of the human βAPP gene, a number of animals are now available. There are also species that develop β-amyloid deposits naturally with age, particularly dogs and nonhuman primates (69-74). Because of their relatively large brains, these animals are particularly well-suited to the development of Aβ-imaging strategies, but their long life-spans and genetic heterogeneity are drawbacks for testing therapies. Transgenic mice, on the other hand, can develop impressive quantities of cerebral β-amyloid deposits, in some cases beginning as early as four months of age.

A number of βAPP transgenic mouse lines have now been produced (75). Three well-characterized models that develop ample cerebral amyloid are the Athena (PDAPP) mouse, the Hsiao (Tg 2576) mouse, and the Novartis (APP23) mouse (76-78). The characteristics, advantages and disadvantages of the various transgenic models of AD have recently been thoroughly reviewed (75). Currently, only the Tg 2576 model is widely available to academic and industrial researchers. This model will be described in more detail, as well as the results of crossed βAPP and presenilin 1-transgenics. It should be borne in mind that the existing transgenic models have a number of interesting and useful characteristics, but none yet fully recapitulates the behavioral and pathological features of AD.

The Hsiao Tg 2576 mice express the human βAPP transgene, with the "Swedish" double mutation K670N-M671L (77). The transgene is driven by a hamster prion protein promoter, and expression is primarily in neurons, but also occurs to a lesser degree in astrocytes. The expression of the transgene is approximately five to six times endogenous mouse βAPP, and remains stable throughout adulthood. Around the age of nine to ten months, Aβ levels in the brain start to rise markedly, and the Tg 2576 mice begin to deposit Aβ in diffuse and compact senile plaques. Occasionally, cerebral blood vessels also are amyloidotic (74). The neocortex and hippocampus are the most affected regions. By sixteen months of age, amyloid deposition can be copious, although variability among animals is high (79). The amyloid that is deposited in the neuroparenchyma is in the form of diffuse and compact senile plaques. In many ways, these plaques resemble those seen in humans with AD. The compact deposits are birefringent after staining with Congo red, are surrounded by abnormal neurites and astrocytes, and are closely associated with microglial cells (80). Homozygous Hsiao mice have not yet been produced. At present, these mice must be maintained on a hybrid background (C57Bl6/SJL), or they will not survive long enough to develop senile plaques. Recently, it has been shown that Hsiao mice crossed with mutant presenilin-1 (PS1M146L) transgenic mice have accelerated deposition of amyloid (81, 82). This observation has also been made with a different line of mutant βAPP/PS1 transgenics (83). These findings indicate a significant interaction of presenilin 1 and Aβ *in vivo*, and provide animal models with more rapid, and possibly less variable, Aβ deposition than in uncrossed βAPP transgenics.

The arrival of transgenic models of cerebral β-amyloidosis is a boon to the AD therapeutic research community. Th small size and short life span of these animals make feasible the relatively rapid and inexpensive testing of inhibitors of Aβ

production and aggregation. Imaging methodology may soon improve to the point where *in vivo* imaging of amyloid load in mice is also possible. Meanwhile, such studies can be profitably undertaken in larger species. A key question that remains to be answered is whether compounds that inhibit Aβ production or aggregation in animal models will impede the pathogenesis of AD. This issue and others regarding the relationship of transgenic models to the human disease should soon be resolved.

Conclusion – Over the past few years, there has been significant progress in the field of Alzheimer's disease, especially in the areas of biophysical techniques to assist with the elucidation of the actual chemical targets, and in the development of the transgenic mouse models. Continued efforts in these areas, as well as towards the identification of potential chemical inhibitors, should assist in bringing forth potential drug candidates for clinical evaluation in the AD arena.

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